Hum Genet (1989) 83: 1–15

APPENDIX 4

Human Genetics

© Springer-Verlag 1989

Review articles

The polymerase chain reaction: an improved method for the analysis of nucleic acids

Hans-Peter Vosberg

Max-Planck-Institut für medizinische Forschung, Jahnstrasse 29, D-6900 Heidelberg, Federal Republic of Germany

Summary. The polymerase chain reaction (PCR) is a method for the selective amplification of DNA or RNA segments of up to 2 kilobasepairs (kb) or more in length. Synthetic oligonucleotides flanking sequences of interest are used in repeated cycles of enzymatic primer extension in opposite and overlapping directions. The essential steps in each cycle are thermal denaturation of double-stranded target molecules, primer annealing to both strands and enzymatic synthesis of DNA. The use of the heat-stable DNA polymerase from the archebacterium *Thermus aquaticus* (*Taq* polymerase) makes the reaction amenable to automation. Since both strands of a given DNA segment are used as templates, the number of target sequences increases exponentially. The reaction is simple, fast and extremely sensitive. The DNA or RNA content of a single cell is sufficient to detect a specific sequence. This method greatly facilitates the diagnosis of mutations or sequence polymorphisms of various types in human genetics, and the detection of pathogenic components and conditions in the context of clinical research and diagnostics; it is also useful in simplifying complex analytical or synthetic protocols in basic molecular biology. This article describes the principles of the reaction and discusses the applications in different areas of biomedical research.

Introduction

The extent to which genetic properties or gene activities can be studied in molecular terms depends critically on the availability of DNA or RNA molecules in numbers of copies large enough to warrant analysis by current methods. To give an example: although the cloning of genes involves the manipulation of single molecules, their detection requires subsequent amplification in appropriate hosts. We can estimate that, regardless of the molecular analysis applied, between 10^5 and 10^6 DNA or RNA molecules (or gene copies) must be available for a single analytical test. Only a few specialized protocols, such as in situ hybridization, exist that allow the identification of single copy genes in single chromosomes. Obviously, any increase in physical sensitivity that could improve the resolution by lowering the detection threshold would produce otherwise unavailable information. Quite naturally, strenuous efforts have been made over the years and are still being pursued, to enhance the resolving power of existing methods or to design new techniques with the goal of detecting smaller numbers of molecules.

A different approach for the investigation of nucleic acid sequences has been designed by Saiki et al. (1985). These authors have invented the method of the polymerase chain reaction (PCR), which is not based on increased detection sensitivity but, instead, on the expansion of the number of target sequences, which are then subject to a conventional analysis.

An important aspect of the PCR is that selective amplification of a sequence of interest reduces, at the same time, the background of sequences that are not wanted. This condition facilitates not only sequence detection, but also preparative manipulations of amplified DNA. By eliminating the need for extensive purification, the PCR minimizes the time and labor needed for handling nucleic acids. It may adequately be qualified as a form of "cell-free molecular cloning" (A. Wilson, quoted in Saiki et al. 1988a).

It is foreseeable that the PCR will enhance the power of diagnostic activities that depend on the analysis of DNA or RNA sequences: prenatal diagnosis of inherited disorders, genetic counseling, clinical disease diagnosis, forensic investigations and related topics. In addition, this new method turns out to be very useful in a number of basic research applications in molecular biology and genetics.

The distinct advantages and limitations of this new method are described and discussed in this review article. Typical applications of the PCR, which are already in use or which can be anticipated in human genetics and in other areas of biomedical research will be considered.

Rarely has a new technique in molecular biology and genetics been so successful within such a short time. New and interesting applications are presently being published at a fast rate. Because of the speed of this development, it is not my intention to give a complete description of all conceivable uses of PCR. In addition, detailed experimental protocols are not included. Instead, the reader is referred to Mullis and Faloona (1987) and to Saiki et al. (1988a). The term "amplification" has various connotations, even in the context of molecular genetics. In this article, it is used to describe the process and result of the PCR.

The technique of the PCR

The logic of the reaction is simple in principle. It depends on the annealing of oligonucleotides to homologous DNA or RNA sequences and on enzymatic DNA synthesis in vitro primed by these oligonucleotides. A pair of primers complementary to both strands of a DNA molecule and flanking a

258351

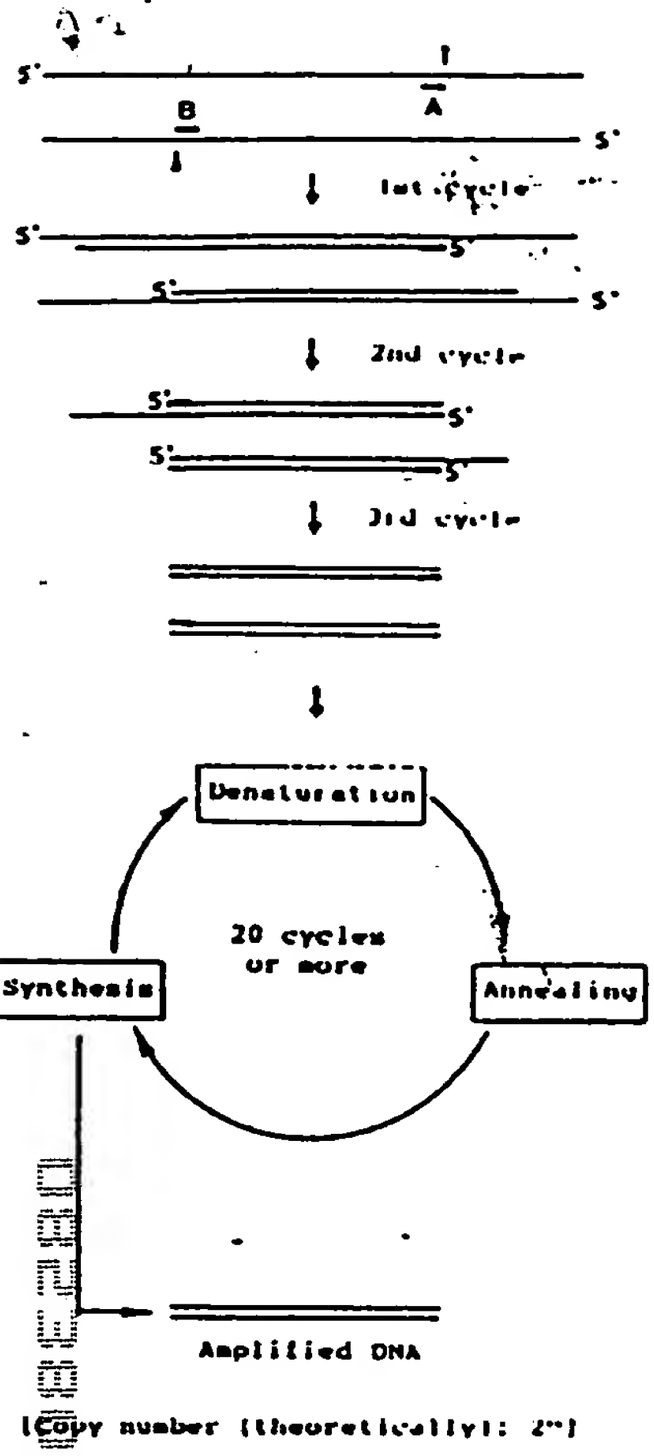

Fig. 1. A simplified scheme of the polymerase chain reaction (PCR) with double-stranded DNA as template. The two primers *A* and *B* serve to initiate DNA synthesis in a defined region. The *arrows* indicate the direction of synthesis in each cycle. Each cycle consists of template denaturation by heat, primer annealing and enzymatic synthesis. DNA products of discrete length (defined by the distance between the 5' ends of the primers; see the *arrowheads* in the *top two lines*) are generated from the third cycle on. Only these molecules are amplified exponentially. The 5' ends of DNA strands used in the first two cycles are indicated to facilitate orientation. The linear increase of molecules with heterogeneous length is disregarded here (*n* number of cycles)

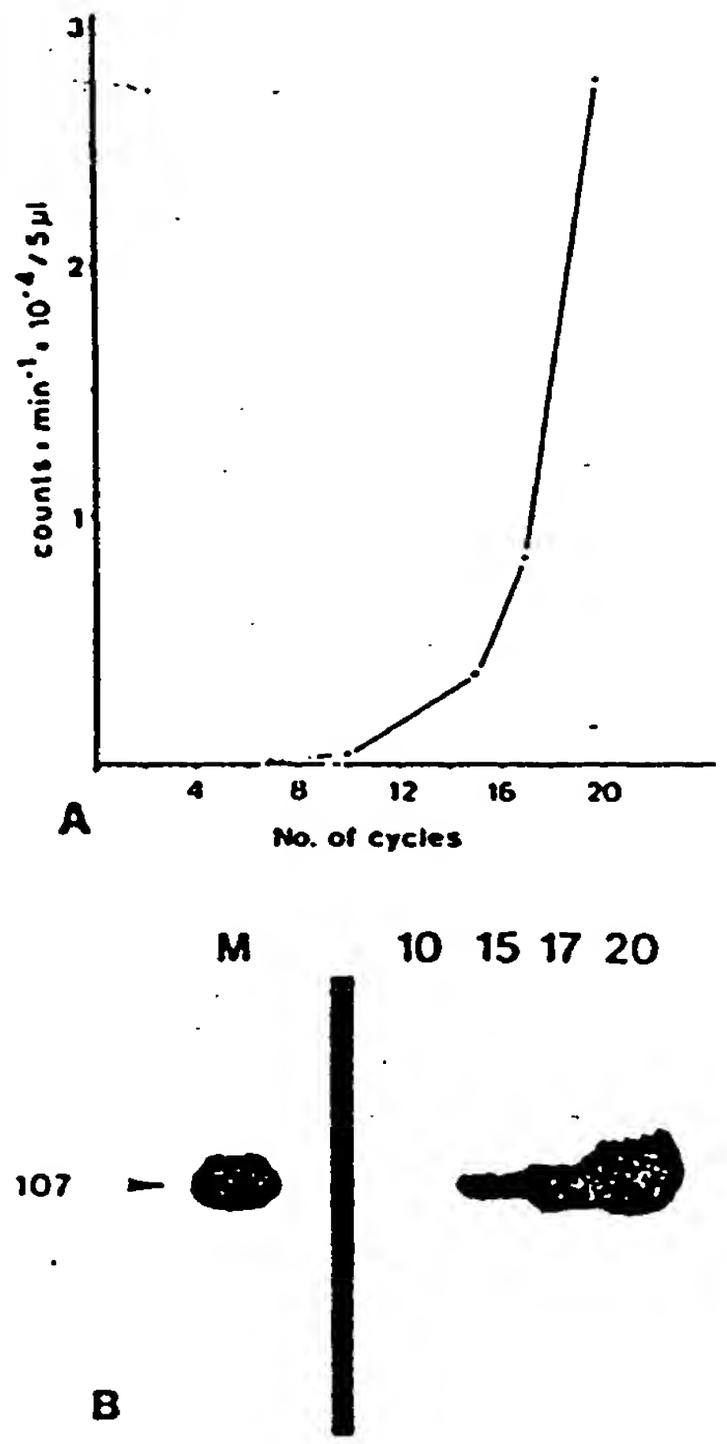

Fig. 2A, B. Demonstration of the exponential increase in the amount of amplified DNA. A 107-bp-long HIV 1 (human immunodeficiency virus 1) specific DNA fragment was amplified by *Taq* polymerase primed by two 20mers in the presence of $\alpha[^{32}P]dCTP$ (in 50 µl assays essentially as in Saiki et al. 1988). Five separate reactions were stopped after 7, 10, 15, 17 and 20 cycles. Aliquots (5 µl) were acid-precipitated (A) and separated on a 8% polyacrylamide gel (see the autoradiographic analysis in B; the 7-cycle reaction was omitted here). The efficiency per cycle in this experiment was about 80%. *M* Size marker. (Courtesy of M. Plorde)

target region of interest is used to direct DNA synthesis in repeated cycles in opposite and overlapping directions. In each cycle, both strands are templates for the generation of two new duplex molecules. This leads (theoretically) to a doubling of the number of target sequences in each round of synthesis. Thus, the overall increase in this number is exponential.

The general course of the reaction with DNA as the initial template is outlined in Fig. 1. Each cycle is initiated by melting double-stranded DNA at 91°–95°C (usually for 1 min) to obtain single-stranded templates. This step is followed first by annealing of the primer oligonucleotides, which are added in large molar excess over template strands, and then by a brief pulse of DNA synthesis (normally between 2 and 5 min). The primers are at the beginning of the reaction in 10^6-fold to 10^{12}-fold stoichiometric excess, depending on the original concentration of target sequences. The temperatures applied for primer annealing (between 50° and 55°C, occasionally below 50°C) and DNA synthesis vary with the enzymes used (e.g., between 58° and 72°C for the heat-resistent *Taq* polymerase) and depend also to some degree on the base composition of the primers. The lower the G + C-content, the lower the optimal temperature for the reaction (Kim and Smithies 1988).

Figure 2 depicts the exponential course of the amplification with the DNA polymerase of *Thermus aquaticus* and a HIV1 DNA sequence as target. This reaction was followed by the incorporation of radio-actively labeled nucleotides and monitored by autoradiographic product analysis.

DNA or RNA as a template may be isolated from any biological source. It can be obtained from cells (Kawasaki et al. 1988; Kim and Smithies 1988), hair roots (Higuchi et al. 1988a), sperm (Li et al. 1988) or surgical biopsy tissue samples (W. Mommaerts, personal communication). Even DNA extracted from embedded archival tissues (Impraim et al. 1987; Smit et al. 1988; Lai-Goldman et al. 1988) or from specimens of extinct animal species (Paabo and Wilson 1988) is suitable for the amplification reaction.

The amplification of RNA sequences (Fig. 3) is preceded by a reverse transcription step, resulting in the generation of a

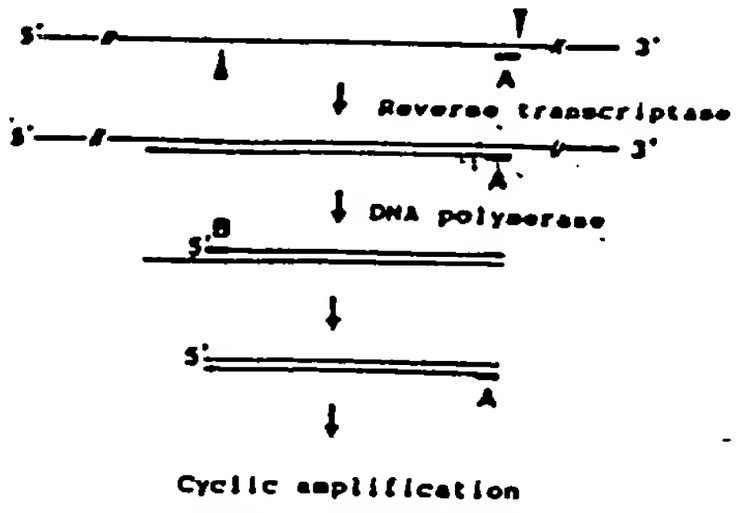

Fig. 3. Scheme for amplification with mRNA as initial template. The first step primed by oligonucleotide *A* is the synthesis of single-stranded cDNA by reverse transcriptase. The conversion into a double-stranded DNA molecule is achieved in a second step with a DNA-dependent DNA polymerase (*Taq* or Klenow) primed by oligonucleotide *B*. The reaction proceeds in cycles as shown in Fig. 1. Double-stranded DNA products of discrete length appear from the third cycle on

single-stranded cDNA complementary to the original RNA (mRNA, viral RNA, etc.). This cDNA is then in a second step converted into double-stranded DNA by the action of the amplifying DNA polymerase.

Different aproaches are available for the reverse transcription of mRNAs. cDNA may be obtained either by oligo(dT) priming (see Todd et al. 1987), by random hexamers (Veres et al. 1987; Noonan and Roninson 1988), or directly by gene specific oligonucleotides (Harbarth and Vosberg 1988). The two former procedures have the strategic advantage that more than one mRNA can be identified using the reverse transcripts present in a single assay mixture.

The reaction with RNA sequences may be complicated by a concurrent amplification of contaminating DNA sequences. Because of the extraordinary sensitivity of the method, small numbers of DNA molecules, otherwise undetectable, could contribute to the products of the reaction. To avoid such complication, DNA can be excluded in a number of ways. DNase (if free of RNase) may digest DNA selectively. It is also possible to remove DNA as an amplification target by restricting it between the primer annealing sites (Harbarth and Vosberg 1988). If mRNA from higher eukaryotes is to be amplified, an obvious measure is the selection of primers that recognize separate exons. In this case, DNA- and RNA-dependent products can be distinguished by their different lengths: products derived from DNA include intron sequences and are therefore longer than those derived from RNA.

Occasionally, template sequences may be refractory to amplification due to stable intramolecular secondary structure within template strands, e.g., if G + C-contents of target regions are high. This complication can be overcome by using the nucleotide analogue 7-deaza-dGTP instead of dGTP (or in addition to dGTP) as a precursor for DNA synthesis. The analogue destabilizes intrastrand folding without impairing Watson-Crick base pairing between strands (McConlogue et al. 1988).

For the synthetic step in the cycle, a number of different DNA polymerases can be applied. The original protocol (Saiki et al. 1985) made use of the Klenow fragment of *E. coli* DNA polymerase I. The unmodified DNA polymerase I, the DNA polymerase of the phage T4 or the modified T7 DNA polymerase can also be applied (Teynor-MacLachlan 1988; Keohavong et al. 1988a, b). A critical disadvantage of these enzymes is their heat lability. Since they do not survive the DNA denaturation temperature (91°–95°C), fresh samples have to be added in each cycle.

The most frequently used enzyme is now the heat-stable DNA polymerase of the archebacterium *Thermus aquaticus*, designated *Taq* polymerase (Chien et al. 1976). This enzyme survives even extended incubation at 95°C (Saiki et al. 1988a). It offers a number of advantages: first, it does not have to be added in each cycle (essentially, with a good enzyme preparation, the addition of one unit is sufficient for the entire amplification running through 30 or more cycles). Secondly, by allowing synthesis at elevated temperature, it reduces the chances of unintended oligonucleotide priming by destabilizing mismatch-pairing with unwanted target sequences, as may result from partial homology with random or related, but not identical sequences. This is particularly important if genes or transcripts, which originate from multigene families, are amplified. Mismatch-priming is less likely to occur at higher than at lower annealing and/or polymerization temperatures (Saiki et al. 1988a). Thirdly, the availability of the heat-stable *Taq* polymerase was a critical prerequisite for the development of automatic equipment for PCRs (see below).

The PCR is efficient, specific and very sensitive. Regarding efficiency, the theoretical upper limit of the number of product molecules is 2^n, where n is the number of cycles. This means that every target sequence present at the beginning could, in 20 cycles, give rise to about a million progeny molecules. Under normal experimental conditions, this value is not obtained, however. A more realistic average efficiency of 85% per cycle (Saiki et al. 1985) reduces the overall yield to a value of about 2.2×10^5 in 20 cycles ($= 1.85^{20}$). The exponential increase in the number of product molecules is not unlimited. One example of conditions contributing to a gradual decrease in the efficiency with increasing cycle number is the increasing amount of template molecules that have to be accepted by an enzyme that at the same time loses some of its activity because of repeated heating. If heat-labile DNA-polymerases are used, the need for adding new enzyme to each cycle leads to a gradual change in the assay composition; this may affect the catalytic activity of the enzyme.

An attractive feature is that in many cases target sequences do not have to be purified extensively prior to amplification.

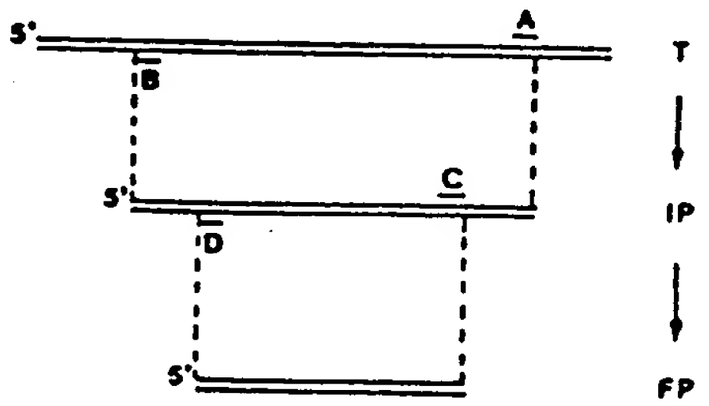

Fig. 4. Amplification with nested sets of primers. To increase the specificity of products obtained from complex template mixtures, the reaction can be started on template *(T)* with the primers *A* and *B*. They define the length of the intermediate product *(IP)*. In a subsequent reaction, this is the template for the primers *C* and *D*, which are located in the region between primers *A* and *B*. The length of the final product *(FP)* is determined by *C* and *D*. Altogether four primers are involved in template selection. Modified versions of this scheme, e.g., with three primers, are conceivable

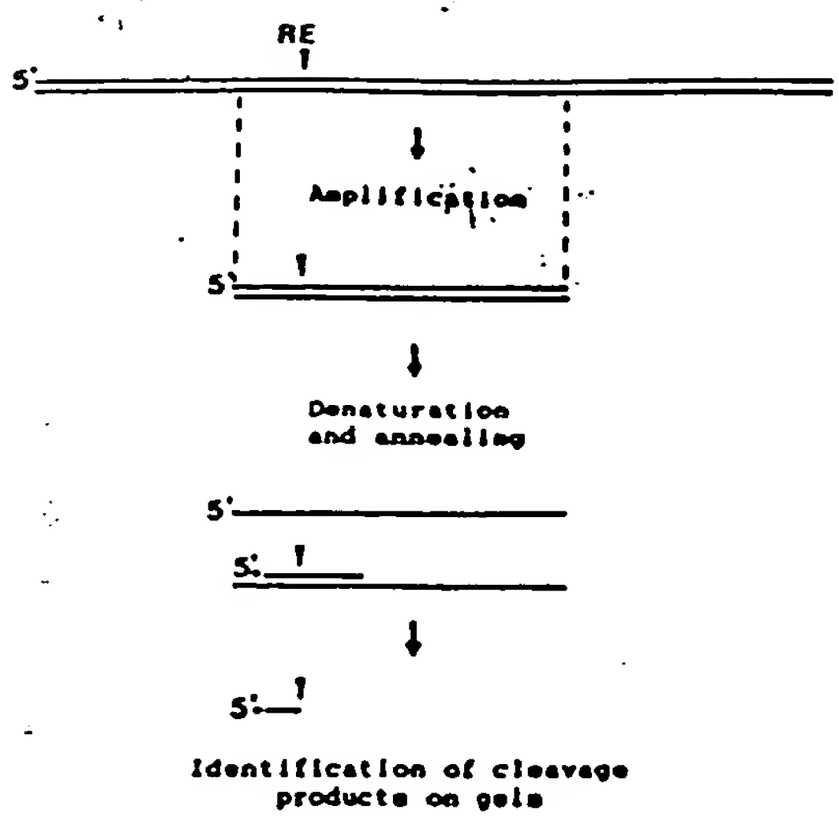

Fig. 5. Oligomer restriction for the identification of amplified DNA. The amplified region includes a cleavage site for a restriction endonuclease *(RE)*. PCR products are denatured and annealed to a 5' end labeled oligonucleotide, which did not serve as primer. After cleavage of the duplex with the appropriate restriction enzyme, the marker fragment carrying the label is identified by gel electrophoresis

The method works on genomic DNA as a whole or on crude mixtures of total cellular RNA (Saiki et al. 1988a; Harbarth and Vosberg 1988). Serum samples can be taken directly as a source of infectious (parasitic) DNA or RNA (Larzul et al. 1988; R. Seelig, personal communication).

However, uncontrolled biochemical sample compositions and a high degree of DNA or RNA complexity have the disadvantage of reducing reaction specificity (and also efficiency). Random primer-target interactions cannot be excluded. Measures to enhance specificity are an increase (as already mentioned) in the annealing temperature, the use of nested sets of primers, or partial fractionation of crude preparations. Nested sets of primers (Fig. 4) involve one round of repeated synthesis with one set of primers and a second round with primer sequences located between the primers used in the previous round (Engelke et al. 1988; Haqqi et al. 1988). This will essentially homogenize the sequence of interest if unwanted background amplifications pose a problem. The use of rapid size fractionation to gain specificity in the amplification of target sequences in complex template mixtures has been reported recently (Beck and Ho 1988).

The amplified products are verified by a number of criteria. The first and most frequently used criterion is the length of the product visualized in ethidium bromide stained agarose or polyacrylamide gels. In many cases the length of an amplified fragment can be anticipated from the known positions of primer annealing sites on known target sequences. Unpredictable lengths may occur in rare cases with introns of unknown composition or if only one of the ends of a DNA or RNA target segment is known. The amplification will then yield new information, which is in itself a useful application of the procedure. Further criteria are predictable restriction sites, which are monitored either directly (Deng 1988; Harbarth and Vosberg 1988) or by a procedure called oligomer restriction (Kwok et al. 1987). The latter method uses radioactively 5'-labeled oligonucleotides. They are annealed to the denatured amplification products and give rise, after cleavage with a defined restriction endonuclease, to labeled fragments of distinct length, a particularly fast, albeit indirect, procedure to identify an amplified product (see Fig. 5).

Alternatively, the products can also be blotted on filters according to Southern (1975) or dotted directly and hybridized with labeled diagnostic oligonucleotides, which anneal to sequences between the primer sites (Saiki et al. 1985; Harbarth and Vosberg 1988). Ultimately, the amplified fragments can be sequenced directly, occasionally even without further purification of the amplified fragments (Saiki et al. 1985; Engelke et al. 1988; Keohavong et al. 1988b; Vigilant et al. 1988). A modified PCR protocol leading to an accumulation of single-stranded DNA in the assay mixture greatly facilitates sequencing (Gyllensten and Erlich 1988; Innis et al. 1988); for details see the section "Applications in basic molecular biology." Direct sequencing is indicated as the standard procedure for the verification of, for instance, polymorphic HLA haplotypes or of genomic mutations (Todd et al. 1987; Scharf et al. 1988; Wong et al. 1987; Simpson et al. 1988).

It is still undetermined where the length-limits are in the amplification of target sequences. Encouraging data were obtained with purified cloned DNA, which could be amplified up to a length of over 2 kb (Saiki et al. 1988a; Kim and Smithies 1988). With genomic DNA a length of 2 kb has been successfully amplified (Keohavong et al. 1988b). Reportedly, longer products can be obtained. We have achieved a 1.7-kb-long myosin heavy chain cDNA fragment, starting with total RNA from muscle tissue (M. Pfordt and K. W. Diederich, unpublished observations). The *Taq* polymerase may be better suited than the Klenow enzyme for the amplification of longer sequences (Saiki et al. 1988a) since its catalytic action is highly processive (Innis et al. 1988) and difficulties due to intramolecular DNA secondary structure are less likely to occur at temperatures optimal for the archebacterial enzyme (60°–70°C).

A very important feature of the PCR is its high sensitivity. Saiki et al. (1988a) reported that a 10^{-6} dilution of genomic DNA containing the β-globin gene into genomic DNA with a homozygous deletion of this gene still allowed amplification of a β-globin target sequence in a reaction over 60 cycles. This result suggests that a target sequence, which is present only once in 10^5 to 10^6 cells, can be detected by amplification. Consequently, single isolated cells or single sperm are suitable for the detection of genomic target sequences (Kim and Smithies 1988; Li et al. 1988). A relatively high sensitivity has also been reported for the detectability of mRNA sequences. Thus, with a conservative estimate of 30000 template molecules (probably less) in unfractionated total RNA preparations from muscle tissue, a β-myosin heavy chain gene fragment was amplified to autoradiographic visibility in 20 cycles with the Klenow enzyme (Harbarth and Vosberg 1988). In another study on muscle-related gene expression, it was observed that a single muscle fiber from an avian skeletal muscle is sufficient for the detection of myosin heavy chain message (B. Kirschbaum and D. Pette, personal communication). A comparably high sensitivity was recently demonstrated in experiments showing that the dystrophin mRNA does not normally appear only in muscle, but possibly also in minute quantities in non-muscle tissues and cells (Chelly et al. 1988). Whether tissue cross-contamination contributed to this unexpected result remains to be seen. On a cellular level, it has been shown that the RNA content of a single cell is sufficient for sequence specific amplification (Rappolee et al. 1988).

The dystrophin mRNA analysis has, moreover, a bearing on another relevant aspect: the PCR can be used to compare mRNA contents of different cells or tissues in at least semiquantitiative terms. The principle of such a comparison relies on co-amplification of two mRNAs, the relative content of one of which is known from independent analysis. In this study, the mRNA coding for aldolase A was used as an internal standard. Alternatively, one could add as a reference a known number of copies of in vitro synthesized cRNA molecules with a sequence similar to that of the tested mRNA.

Since in vitro DNA synthesis is, by its very nature, an error-prone process, sequence fidelity of amplification products is a point of major concern. A number of reports have addressed this question in detail (Scharf et al. 1986; Saiki et al. 1988a; Dunning et al. 1988; Paabo and Wilson 1988). The most extensive assessments have been performed by cloning and sequencing individual amplified fragments derived from regions of the human HLA-DPβ gene (Saiki et al. 1988a) and of the human apolipoprotein B gene (Dunning et al. 1988). In 28 DNA HLA DQβ-clones, each 239 bp long and inserted into a M13-vector and all derived from a single individual, no deletions or insertions were found, although 17 misincorporated bases (mostly transitions) were identified (error frequency: about 0.25%). *Taq* polymerase was used in this experiment, which went through 30 cycles. A similar error frequency was obtained in the study with the apolipoprotein B gene fragments.

The number of misincorporated nucleotides depends on two factors, viz., on the rate of misincorporation during synthesis and on the number of "generations", i.e., the number of synthetic cycles. This number contributes to the error frequency at the end of the overall reaction, since misincorporations occurring in an early "generation" are inflated in number in each subsequent cycle of doubling. The rate of misincorporation *(m)* can be determined by the formula $m = 2f/c$ (Hayes 1968) where f is the frequency of misincorporated bases verified by sequencing and c is the number of cycles. For *Taq* polymerase, the rate was calculated to be about 2×10^{-4} (Saiki et al. 1988a). A slightly lower value for this enzyme (1.1×10^{-4}) was recently reported from a study involving in vitro primer extension synthesis by this enzyme (without amplification) and subsequent genetic screening in vivo for single base substitutions (Tindall and Kunkel 1988).

Preliminary studies with products amplified by the Klenow enzyme suggest a rate of about 8×10^{-5} for this enzyme (Saiki et al. 1988a; Oste 1988). Thus, the Klenow enzyme appears to be somewhat more reliable in preserving DNA sequences during the process of amplification. The observed rate is, however, relatively high compared with data obtained under conditions of only one round of DNA synthesis (Tindall and Kunkel 1988). In the latter study, the Klenow enzyme had a 4- to 8-fold lower rate of errors than the *Taq* polymerase.

The two enzymes differ in their ability to catalyze 3'–5' exonuclease proofreading. While the Klenow enzyme is endowed with this ability, the *Taq* polymerase is not (Chien et al. 1976; Tindall and Kunkel 1988). This difference, possibly together with the mutagenic effects of high reaction temperatures (see Drake and Baltz 1976), may at least partially explain the relatively high rate of misincorporations by the *Taq* polymerase.

This complication is for most purposes of no consequence for the PCR. Analytical procedures such as direct sequencing or filter hybridization with allele-specific oligonucleotides do not suffer from the small number of errors in the amplified products (up to 1 in 400 bp are wrong, with a random distribution). However, if cloning of individual fragments is required, sequences need confirmation by analyzing independent isolates, in particular if only a few copies of the target sequences were initially available. It therefore seems advisable that experiments involving cloning of amplified DNA should be carried out with a large rather than a small number of template molecules, and amplification should take place in as many cycles as are necessary, but not more. Although the *Taq* polymerase is generally preferable because of its heat stability, the less convenient Klenow enzyme or the phage T4 or T7 DNA polymerases with their higher fidelity rate (Tindall and Kunkel 1988; Kuchta et al. 1988) may in exceptional cases still be useful.

It has already been mentioned that the heat stability of the *Taq* polymerase makes reactions with this enzyme amenable to automation. Since in many cases one or at most two additions of enzyme are sufficient to maintain a high rate of synthesis, equipment is needed that is able to regulate temperature changes automatically. A number of relatively expensive machines are available, but cheap and simple laboratory solutions have also been proposed (Rollo et al. 1988; Foulkes et al. 1988; Kim and Smithies 1988). We have constructed an inexpensive computer-controled minirobot (a portal robot), which acts by transferring the incubation vials in a cyclic fashion from one glycerol bath (for DNA synthesis) to a second (for denaturation) and then to a third one (for primer annealing). Each bath has a different preset temperature. The vials are for each incubation completely submerged by the robot lever. Thus, the temperature transition between the reaction steps is fast, and losses of volume due to evaporation are avoided (M. Pfordt and K. W. Diederich, unpublished results).

The inherent high sensitivity of the PCR requires a special comment regarding experimental care, which may affect the interpretation of results. Minute contaminations of benches or frequently used laboratory equipment with template molecules from various sources, including previous amplifications, may lead to the inadvertent addition of target sequences in assay mixtures and, hence, to false-positive "signals" (Simpson et al. 1988; Lo et al. 1988b; Kim and Smithies 1988). In the course of our experiments, this problem arose occasionally with a certain time lag after the introduction of a new set of primers. This complication, which may be regarded as an unwanted demonstration of the power of the polymerase chain reaction, requires extreme care in the handling of all reaction components and regular quality checks in the form of negative control amplifications, i.e., reactions without added template. Experimental conditions, such as sterile working habits, may even be advisable.

Applications of the polymerase chain reaction

Genetic and related research

Essentially, three major areas of biomedical research will benefit from PCR. These are human genetics, including genetic services and certain forensic applications, clinical investigations with the goal of monitoring the causes of disease, the progression of disease and therapeutic success and, finally, basic molecular biology. Table 1 depicts a list of applications

Table 1. Applications of the polymerase chain reaction

1. Genetic research and counselling
Detection of mutations
Prenatal diagnosis of inherited disorders
Prenatal sex determination
Carrier detection in families and populations
RFLP linkage studies
Generation of probes for gene mapping and in situ hybridization
Population genetics
Forensic identification of individuals
2. Clinical investigations
Pathogen detection and typing
Identification of activated oncogenes and tumor typing
Monitoring of disease progression and therapy
Disease susceptibility studies and preclinical risk assessment
3. Applications in molecular biology
DNA sequencing
Gene synthesis and gene modification
Gene expression studies
Gene targeting
Site directed mutagenesis

that have already been reported in the literature or which will presumably be realized soon.

The usefulness of the PCR for genetic research was documented in the first PCR report, in which probing of the sickle-cell mutation in amplified DNA fragments was demonstrated (Saiki et al. 1985). Soon thereafter, it was shown that direct sequencing of amplified mutant alleles is feasible. This study included the identification of previously unknown mutations of the β-globin gene (Wong et al. 1987).

For the routine analysis of mutants, allele-specific oligonucleotides (ASO) (Connor et al. 1983) can be used in a simple "dot-blot" filter hybridization assay that affords a rapid distinction of homozygous and heterozygous constellations (see Fig. 6, taken from Saiki et al. 1986). The detection probes were radioactively labeled in most cases. However, the application of non-radioactive biotinylated or enzyme-labeled probes has also been reported (Bugawan et al. 1988; Saiki et al. 1988b; Syvänen et al. 1988; Lo et al. 1988a). Isolation and labeling of DNA probes can be achieved by PCR in a single preparative step (Liang and Johnson 1988; Lo et al. 1988a). Alternative methods for the identification of amplified globin alleles are the oligomer restriction technique (Embury et al. 1987) or direct restriction mapping of amplification products (Chehab et al. 1987; Kulozik et al. 1988).

The application of the PCR for prenatal diagnosis and mutation analysis of globin-related inherited disorders has frequently been described (Chehab et al. 1987; Embury et al. 1987; Cai et al. 1988; Kulozik et al. 1988; Bugawan et al. 1988; Chan et al. 1988; Saiki et al. 1988b). Fetal DNA was prepared either from amniocytes or from chorionic villi. An interesting prospect is the use of sets of appropriately chosen oligonucleotides, which allows the rapid identification of multiple mutations within a given population or geographic area (Cai et al. 1988; Diaz-Chico et al. 1988).

Another genetic trait studied by DNA amplification is the α_1-antitrypsin deficiency. Adult individuals carrying the MM, MZ, or ZZ alleles (Bruun Petersen et al. 1988; Newton et al. 1988), or fetuses at risk of α_1-antitrypsin deficiency (Abbott et al. 1988) could readily be identified by appropriate ASOs. In the latter case, chorionic villus DNA was amplified and diagnostic use was made of a known polymorphic restriction site in exon III of the α_1-antitrypsin gene. In this analysis, probe hybridization was not required.

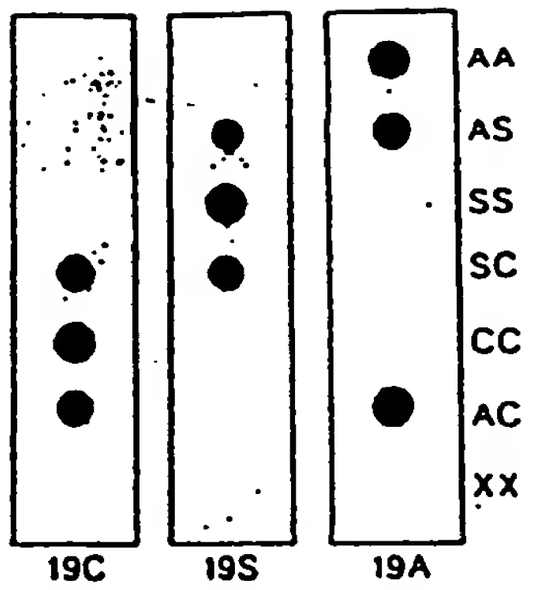

Fig. 6. Example of an analysis of homo- and heterozygosity in genomic DNA by allele specific oligonucleotides (ASO). The ASO probes were designed to recognize the hemoglobin allele C *(19C)*, the sickle cell allele S *(19S)* and the normal β-globin allele *(19A)*. Portions of genomic DNA (1 μg) with known β-globin genotype were amplified with the Klenow enzyme. Aliquots of PCR products were denatured and applied to a nylon filter. The ASO probes were labeled at their 5' ends with [^{32}P]. After hybridization, the filters were washed for 10 min at 55°C (for 19C) or at 60°C (for 19S and 19A). The homo- and heterozygous genotypes are indicated on the *right side*. *XX* is DNA from a cell line with a homozygous β-globin gene deletion. (For details see Saiki et al. 1986; reproduced with permission)

If the disease-causing mutations are known, allele-specific amplification may be used for screening not only sibships carrying certain recessive traits, but even populations. Carriers without a family history of the disease could then be detected. DiLelle et al. (1988) have discussed such a measure for the identification of PKU carriers and suggested that it is technically feasible. This conclusion is corroborated by PCR-based genotyping of apolipoprotein E alleles in a cohort of 68 individuals (Weisgraber et al. 1988), by studies on the genetic susceptibilty to insulin-dependent diabetes mellitus (Gu et al. 1988) and also by an investigation of the spectrum of β-thalassemia genes in Spain (Amselem et al. 1988).

If the mutations leading to an inherited defect are not known, the PCR may provide the missing information by amplifying genomic DNA or mRNA sequences (Simpson et al. 1988; see also the globin mutants reported by Wong et al. 1987). Using the combination of amplification and direct sequencing of amplified DNA, a point mutation in the HPRT gene $HPRT_{Munich}$ (which causes gouty arthritis) could be verified. The mutant is a C–A transversion, substituting a serine by arginine at amino position 103 (Cariello et al. 1988). (This substitution was known from protein analysis). In the gene coding for the clotting factor VIII, a previously unidentified missense mutation (a G–C transversion) has been detected by this procedure (Levinson et al. 1987). More recently, a mutation in the factor IX gene was also characterized by PCR (Denton et al. 1988).

A powerful combination for a rapid mutational analysis is the use of ribonuclease A (RNase A) protection (Myers et al. 1985) together with PCR. The mutational site is first localized by RNase A protection before it is subsequently amplified using the respective genomic DNA or mRNA sequences as templates. This protocol avoids the slow process of genomic library construction and screening. The method was first used

by Veres et al. (1987) for a molecular analysis of the murine sparse fur mutation resulting from a deficient ornithine transcarbamylase. This mutation serves as a model for the most common human urea cycle disorder. A second report based on this strategy was concerned with a deficiency in ornithine-δ-transcarbamylase, causing gyrate atrophy of the choroid and the retina (Mitchell et al. 1988).

The PCR technique has been applied to the analysis of genomic deletions, as they are frequently found in Duchenne/Becker muscular dystrophy patients (Koenig et al. 1987). The detection of previously unknown deletions by scanning genomic DNA with selected multiple sets of primers and the prenatal identification of deletions known to exist in heterozygous carrier mothers will be greatly facilitated by amplification (Chamberlain et al. 1988). The same procedure should distinguish the carrier status of daughters of identified carrier mothers.

The diagnosis of a wide variety of inherited disorders is based on restriction fragment length polymorphisms (RFLP). The presence or absence of a variable cleavage site tags mutations in pedigrees. The reliability of predictions depends on the distance between the marker locus and the gene of interest: the tighter the linkage, the higher the probability that a prediction is correct (for reviews see Botstein et al. 1980; Gusella 1986). The usual method for the identification of mutations via linkage analysis of this type requires sufficient quantities of relatively intact genomic DNA (approximately 1–10 μg per assay), cleavage, gel electrophoresis, Southern blotting and probing RFLP haplotypes with radioactive DNA. The PCR offers a convenient alternative to this time-consuming and costly protocol by amplifying the regions, which include the polymorphic sites, from minute amounts of genomic DNA (1 ng or less). The presence or absence of a diagnostic site is shown directly by cleavage of the amplified DNA fragment. In cases of heterozygosity, both alleles can be identified (see Feldman et al. 1988). The only prerequisite is knowledge of the DNA sequence surrounding the polymorphic sites. To obtain these sequences requires the effect of cloning (mostly of cosmid size fragments), subcloning and sequencing. So far, only a few sites have been sequenced, but this number will certainly increase in the near future.

The first successful application of this new approach involved hemophilia A (Kogan et al. 1987). Polymorphic restriction sites from within the factor VIII gene were used. DNA for prenatal diagnosis was extracted from chorionic villi. The authors claim realistically, as do others (Williams et al. 1988), that a diagnostic result is available within a day, given the appropriate experience. Similar results have been obtained with the prenatal diagnosis of cystic fibrosis (Feldman et al. 1988). In these latter cases, linked extragenic marker sites were used. The genes responsible for these disorders are not known.

A particularly interesting application in a related context is the genotyping of single sperm. Each sperm is the product of a single meiotic event; hence, many such events can be investigated with material obtained from one individual. In an introductory study, it was shown that separate genetic loci can be analyzed simultaneously by DNA amplification (Li et al. 1988). This application should allow the measurement of recombination over distances that are shorter than those covered by pedigree analysis, in particular if recombinational hot spots are involved. It is conceivable that the accurate ordering of tightly linked RFLPs will be greatly facilitated. The use of single sperm may pave the way for a new approach of generating genetic maps for species that are not available for selective breeding.

The PCR has also been used for prenatal sex determination by the amplification of Y chromosome specific DNA sequences (Kogan et al. 1988).

Other genetic research where the PCR has been employed as an improved method are genetic epidemiology (including pharmacogenetics) and population genetics. Interindividual differences in the susceptibilities to environmental components (e.g., alcohol, drugs, pollutants of various kinds) will be amenable to rapid analysis by DNA amplification as soon as the genes involved in the responses to environmental challenges have been identified. The feasibility of using this technique has already been demonstrated by genotyping human class I alcohol dehydrogenase (ADH) alleles. The method allows different allelic variants to be distinguished and thus provides a means for the determination of the ADH isoenzyme pattern of humans (Gennari et al. 1988). This study includes a reliability test by comparing the results obtained by ASO hybridization with isoenzyme variants isolated from liver specimens.

In connection with phylogenetic investigations of human populations, length mutations as well as conformational mutations in human mitochondrial DNA have been determined by direct sequencing of amplified DNA (Wrischnik et al. 1987; Vigilant et al. 1988). Two primers were used for amplification and a third for sequencing. Taking this report into consideration, together with the ease both of sampling DNA-containing specimens (one hair is sufficient) and of handling large numbers of assays, one may anticipate that molecular studies on human populations can be designed on a larger scale than before.

The forensic identification of individuals relies on the demonstration of interindividual genetic differences. Genotyping of people is possible using genomic DNA and probes, most notably those derived from minisatellite DNA sequences (now called variable number tandem repeats or VNTR), which recognize highly variable RFLPs (Jeffreys et al. 1985). However, this approach requires more DNA than can frequently be obtained from relevant biological materials. Single hairs have therefore been taken to extract DNA for a PCR analysis. A freshly plucked hair yields about 200 ng DNA, a shed hair 10 ng; 1 ng is obtained if the hair is very old (Higuchi et al. 1988a). Mitochondrial DNA, which has extensive sequence polymorphism in its D-loop (Aquadro and Greenberg 1983), HLA genes (Higuchi et al. 1988a), single VNTR sequences (Jeffreys et al. 1988) and possibly others can be used as polymorphic markers. Obviously, collecting hairs for genetic analysis may be a convenient alternative to collecting samples that are either difficult to obtain or delicate to handle, in particular if transport over long distances is needed.

Clinical research applications

Extensive use of the PCR can be expected in disease-related clinical investigations and diagnostics. Since in many common diseases, genetic factors (e.g., somatic mutations, inherited disease susceptibilities or multifactorial inheritance) are involved, this research increasingly has cross-connections to classical and molecular genetics. The growing trend to extend diagnostic efforts to the analysis of DNA or RNA sequences underlines the impact that genetic research has on medical research in general.

Malignant and infectious diseases were among the first clinically relevant topics for which the advantages of the PCR were realized. The *ras* proto-oncogenes, which in mammalian organisms form a family of at least five closely related non-allelic isogenes (two being pseudogenes), acquire their transforming potential by point mutations in the amino-acid codons 12, 13 or 61 (for review see Marshall 1986). The standard assay used so far to detect mutated *ras* genes is transfection of NIH-3T3 cells. This method is too laborious for routine analysis of DNA from tumor specimens of patients or experimental animals. To make testing faster and at the same time more sensitive, a dot-blot screening procedure for mutated *ras* genes has been devised on the basis of in vitro amplification with primers flanking the suspected mutation sites and allele (or mutation)-specific oligonucleotides (Verlaan-de Vries et al. 1986). The diagnostic value of the new technique has been confirmed in numerous reports from different laboratories (Bos et al. 1987; Kozma et al. 1987; McMahon et al. 1987; Janssen et al. 1987a, b; Farr et al. 1988; van't Veer et al. 1988; Lyons et al. 1988).

Oncogenes, including *ras*, with structural changes in their activated state lend themselves to this type of analysis since DNA sequence alterations are easily detected. To monitor a change in the transcriptional activity of an otherwise non-mutated oncogene may not be as simple, but will probably also be possible. Thus, it may be expected that the amplification technique will facilitate tumor typing and tumor progression analyses significantly. This includes the possibility of monitoring success or failure of tumor therapy at the molecular level, including the detection of drug resistance in tumors (Layton et al. 1988; Kashanisabet et al. 1988).

The PCR has also been used for the diagnosis of chronic myeloid and acute lymphocytic leukemias that result from a t(9;22) chromosomal translocation (Kawasaki et al. 1988). The fusion leads to the expression of a leukemia-specific chimeric mRNA, which combines information of the ABL proto-oncogene on chromosome 9 with the "breakpoint cluster region" gene (BCR) on chromosome 22. Visualization of this abnormal mRNA relies on oligonucleotide primers located on either side of the junction and on diagnostic oligonucleotides comprising breakpoint/junction sequences. The procedure is very sensitive: 1 pg total cytoplasmic RNA from a leukemia cell line is sufficient for the generation of a tumor-specific DNA fragment by amplification. Since only processed RNA sequences contribute to the appearance of this fragment, genomic DNA does not interfere with the analysis.

The detection of chromosomal translocations can also be achieved by amplifying genomic DNA regions flanking the crossover sites. This has been shown for the translocation t(14;18), which is characteristic for follicular lymphomas (Lee et al. 1987; Creszenzi et al. 1988). The high sensitivity of the PCR affords detection of DNA from 1 in 10^5 cells, permitting a diagnosis under conditions that make the application of morphological, cytogenetic or even molecular analysis (Southern blots) difficult or impossible.

Pathogen detection is the second clinical topic that already benefits from the new methods, most notably in cases where conventional techniques are not sensitive enough or too cumbersome. One such case is the human papilloma virus (HPV), which has been associated with neoplastic change in the cervix (Howley 1987). To facilitate and expand HPV typing (>46 different types are known), the PCR has been adopted for the identification of the virus even in paraffin-embedded tissue (Shibata et al. 1988). Furthermore, human retroviruses can readily be recognized by amplification. The human T-cell lymphoma virus type I (HTLV-I) (Bhagavati et al. 1988; Duggan et al. 1988; Kwok et al. 1988) and the human immunodeficiency virus (HIV) (Kwok et al. 1987; Farzadegan et al. 1988; Murakawa et al. 1988; Byrne et al. 1988) were reliably detected by the new methods. Since proviral DNA sequences (Ou et al. 1988) and viral RNA sequences (Byrne et al. 1988; Murakawa et al. 1988) can be amplified separately, it may be possible to distinguish between latent and proliferative stages of infection and, hence, to monitor disease progression.

Another clinically important virus is the hepatitis B virus, which has been detected in serum samples by in vitro amplification (Larzul et al. 1988).

An important application of the PCR is concerned with the analysis of genetic polymorphisms involved in disease susceptibilities. Endeavors to understand the mechanisms of complex polygenic human diseases and to recognize them while still in a preclinical state have been concentrated on the identification of DNA and protein markers associated with autoimmune disorders or hypertension and coronary heart diseases in families and populations. The specific advantage of the PCR is (as in prenatal diagnosis or carrier status determinations) the greatly facilitated assessment of haplotype sequences known or suspected to be involved in the expression of a disease phenotype. These sequences may serve as markers indicating that a risk of disease exists or, alternatively, they may be important because they contribute directly to the pathogenic mechanisms.

One group of genes studies in this context contains the HLA class II genes (for review see Kaufman et al. 1984). They code for dimeric cell-surface glycoproteins, which with a few exceptions are highly polymorphic. They are normally expressed on the surface of B cells and interact with T cell receptors and antigens to activate T cells and immune responses to antigens. The feasibility of a PCR-based approach to study specific HLA class II haplotypes of patients and of control probands by amplifying and direct sequencing has so far been demonstrated in three reports. They were concerned with the susceptibility to insulin-dependent diabetes mellitus (Todd et al. 1987; Gu et al. 1988) and the dermatologic disorder *pemphigus vulgaris* (Scharf et al. 1988). In both analyses, it was found that the disease susceptibility is largely dependent on the identity of the amino acid residue at position 57 of the DQβ allele of the HLA class II gene cluster. Different routes for the analysis were chosen in these studies: the amplified template sequences were either mRNA (Todd et al. 1987) or genomic DNA (Scharf et al. 1988; Gu et al. 1988).

Other disease-related polymorphisms can also be studied by this method. Thus, the genotype of proteins involved in the cholesterol metabolism defines different degrees of risks, among them that of premature atherosclerosis and coronary heart disease (Berg 1986; Weisgraber et al. 1988). It can be expected that by screening large groups of probands preclinical risk assessment based on PCR-mediated genomic analysis will be a real possibility in the future, at least for some common diseases.

Applications in basic molecular biology

In addition to the prevailing diagnostic use in genetic and clinical research, the PCR serves as a tool to facilitate complex

protocols in basic molecular biology. Small- and large-scale sequencing of DNA, in vitro synthesis of genes, site-directed recombination in vivo, the modification of DNA sequences in vitro or the rapid preparation of DNA probes, gene expression studies and other related activities profit from the power and improved sensitivity of this method. Only a few analytical and preparative applications will be considered here. Many more are conceivable or have already been reported.

Obviously, the PCR can be coupled with DNA sequencing protocols. In numerous reports, the feasibility of direct sequencing of amplified DNA has been demonstrated (Wong et al. 1987; Levinson et al. 1987; Wrischnik et al. 1987; Todd et al. 1987; Engelke et al. 1988; Oste 1988; Cariello et al. 1988; Scharf et al. 1988). The relevant message emanating from these experiments is that minute quantities of DNA or RNA are sufficient for sequencing and that cloning is not required. In most cases, the dideoxy chain termination method was applied (Sanger et al. 1978). Alternatively, the chemical cleavage method (Maxam and Gilbert 1980) could be used, provided one of the amplification pimers carries a 5′ radioactive label.

A substantial improvement of the sequencing protocols is the recently introduced "asymmetric" PCR, which leads to the generation of single stranded DNA by using unequal stoichiometric amounts of the two amplification primers (e.g., 50 pmol for one primer and 0.5 pmol for the second primer; see Gyllensten and Erlich 1988; Innis et al. 1988). The asymmetric reaction proceeds conceptually in two steps, starting with a regular exponential amplification of double-stranded DNA as long as the limiting primer is available. Once this primer has been used up, the excess primer initiates synthesis of single-strands in a linear pogression. This DNA can be sequenced directly with the *Taq* polymerase (Innis et al. 1988) or with the modified T7 DNA polymerase (Sequenase) (Gyllensten and Erlich 1988). Sequencing of single strands avoids difficulties which often result from rapid reannealing of complementary strands or from adventitious homology of sequencing primer regions pesent on both strands.

It has also been suggested that amplification of DNA and RNA molecules be combined with in vitro transcription by appending a promotor sequence to the amplified DNA. This modification offers the opportunity of making single-stranded RNA molecules in vitro in large quantities for subsequent dideoxy sequencing with reverse transcriptase (Stoflet et al. 1988; Sarkar and Sommer 1988).

A novel procedure for DNA sequencing that has been connected with amplification is based on the incorporation of deoxynucleotide analogues carrying a phosphorothioate substitution in the α-position (abbreviated dNTPαS). DNA molecules containing these analogues are specifically cleaved at the phosphorothioate positions in the presence of 2-iodoethanol or 2,3-epoxy-1-propanol (Gish and Eckstein 1988; Nakamaye et al. 1988). Modified DNA is synthesized in vitro by PCR in four separate polymerization reactions (each with a different dNTPαS). Radioactive [^{32}P] label is introduced in a strand-specific manner, e.g., by amplifying with one unlabeled and one 5′-labeled primer. Since both synthesis and partial cleavage are fast and easily achieved, the combination of amplification with this new sequencing protocol may significantly enhance the rate of DNA sequence determination.

Most PCR sequencing experiments performed so far have been based on known sequences flanking the region of interest. This type of analysis is mainly targeted at point mutations

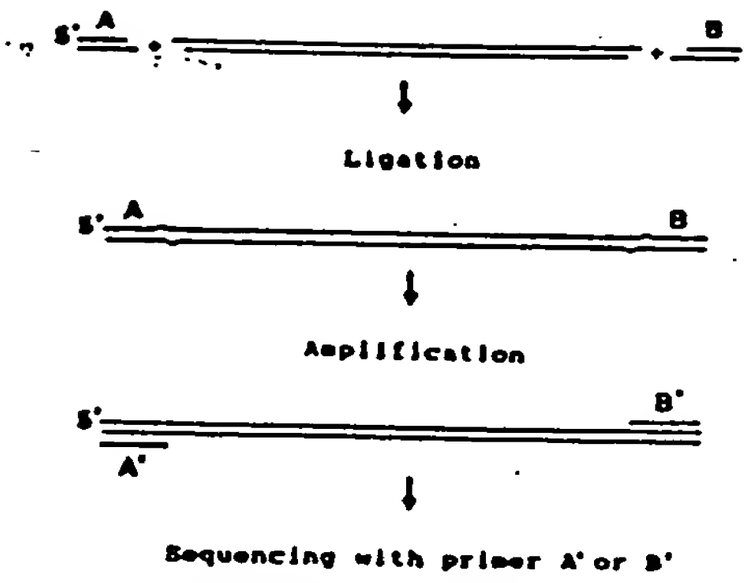

Fig. 7. A PCR-based strategy for the determination of completely unknown DNA sequences. Prerequisites are DNA fragments (400–600 bp long) with non-identical recessed ends (restriction enzyme cleavage sites). Double-stranded oligonucleotide adaptors (*A* and *B*) are ligated to the fragments. After removal of unused adaptor molecules, the fragments are amplified using single-stranded adaptor oligonucleotides as primers (primer *A′* and *B′*). The subsequent separate sequencing of both strands of the PCR product is achieved using either primer *A′* or primer *B′*

or other small sequence alterations, the closing of gaps (about 600 bp or more) in connection with the sequencing of long genes, or the sequencing of unknown introns between exons derived from cDNA. (If sufficient amounts of genomic DNA are available, conventional "walking primer" protocols without amplification could, of course, also be used for the latter purposes.)

The PCR procedure can be applied for the amplification of sequences that lie outside the boundaries of known regions. One approach, designated "inverted" PCR, is based on inversion of the sequence of interest by in vitro circularization and reopening at a different site within the known region. The cleavage results in two known sequences flanking an unknown region. Using the known sequences as anchor for amplification, sequence determinations may be extended to previously undetermined regions (Triglia et al. 1988).

Another, somewhat related approach for amplifying (and sequencing) entirely unknown regions involves ligation of adaptor oligonucleotides to the ends of DNA restriction fragments (isolated, e.g., from cosmids; for the rationale see Fig. 7). The adaptors serve as annealing sites for amplification primers and subsequently also for priming sequencing reactions. To allow sequencing of both strands of one amplified DNA molecule, the 5′ and 3′ adaptors have to be different. Therefore, DNA fragments framed by non-identical staggered restriction sites are preferable. DNA quantities in the nanogram range will suffice to initiate amplification and sequence determination (M. Pfordt, unpublished results).

The investigation of gene expression by monitoring the presence, appearance or disappearance of mRNAs in a few cells will presumably become a major application of the PCR. Some of the conventional methods (Northern, S1 nuclease protection mapping) that monitor gene activities on the level of transcription may soon be replaced, at least to some extent, by the more sensitive new technique. The PCR-based transcript identification has appropriately been designated as "mRNA phenotyping" (Rappolee et al. 1988). This will eventually include the analysis of complex splicing patterns.

A particularly striking demonstration of the potential to unravel new details about transcription and RNA processing

by PCR was the identification of the translational stop codon in the mRNA that codes for the apolipoprotein B48 in intestinal cells. This stop codon is not found in the respective gene. It has been suggested that it results from a tissue-specific co- or post-transcriptional modification of the primary transcript (Powell et al. 1987).

The verification of gene targeting may be mentioned as an additional interesting research application of PCR. The demonstration of homologous recombination between DNA introduced into recipient cells and endogenous genes requires either that a selectable phenotype is induced by the event or that a powerful screening procedure is available (see Kim and Smithies 1988, and references cited therein). The few successful targeting experiments reported so far were mostly based on phenotype selection. Screening was also used, but required laborious and time-consuming manipulations (Smithies et al. 1985). With the availability of the PCR, detection of recombinants is greatly facilitated even if homologous recombination is rare, as is normally the case. Detection is based on the demonstration of amplified DNA of predicted length, which can only be obtained from recombinants and not from non-recombinants or non-homologous recombinants. Oligonucleotides selected from regions flanking insertion sites are used in a manner similar to that employed in the amplification of chromosomal translocation breakpoint/junction regions. It was shown in a test system with a previously established HPRT gene-modified recombinant cell that single cells are amenable to recombination analysis by PCR. Since cell mixtures can easily be investigated, sib-selection protocols were applicable for the identification of rare events in large numbers of potential target cells (Kim and Smithies 1988).

Regarding preparative goals, an important aspect of the PCR is the possibility of combining amplification with directed modifications of target sequences, notably at the ends of regions of interest. Primers carrying additional sequences not present in the original template (promotors, restriction sites, other recognition sequences) at their 5' ends are unaffected in their ability to initiate a strand extension reaction. From the second cycle on, the additional sequences are converted into a double-stranded form and participate in further amplification as if they were part of the original target region. Thus, simply by the mechanism of the PCR, DNA sequences may be altered, in this case by adding new sequences without the need of separate synthetic or enzymatic manipulations.

By analogy, the polymerase chain reaction has also been used for the introduction of base-specific mutations into amplified DNA. Such modifications require primers, which mismatch with a target gene segment. The mismatch is converted into a point mutation (or a small insertion or deletion) by the repeated cycles of DNA synthesis (Rochlitz et al. 1988; Higuchi et al. 1988b). These mutations are located at the ends of amplified DNA, but they can be relocated into the middle of a larger segment by the PCR mediated in vitro "recombination" of two overlapping fragments that carry the same point mutation within the region which is common to both fragments (for details see Higuchi et al. 1988b).

DNA amplification has also been used for the synthesis of DNA molecules several hundred base-pairs in length (Mullis et al. 1986). Overlapping oligonucleotides (74 bases in the reported example) were mutually extended at their respective 5' ends in the initial round of amplification. The resulting double-stranded DNA molecules were then expanded further by additional amplifications using new oligonucleotides,

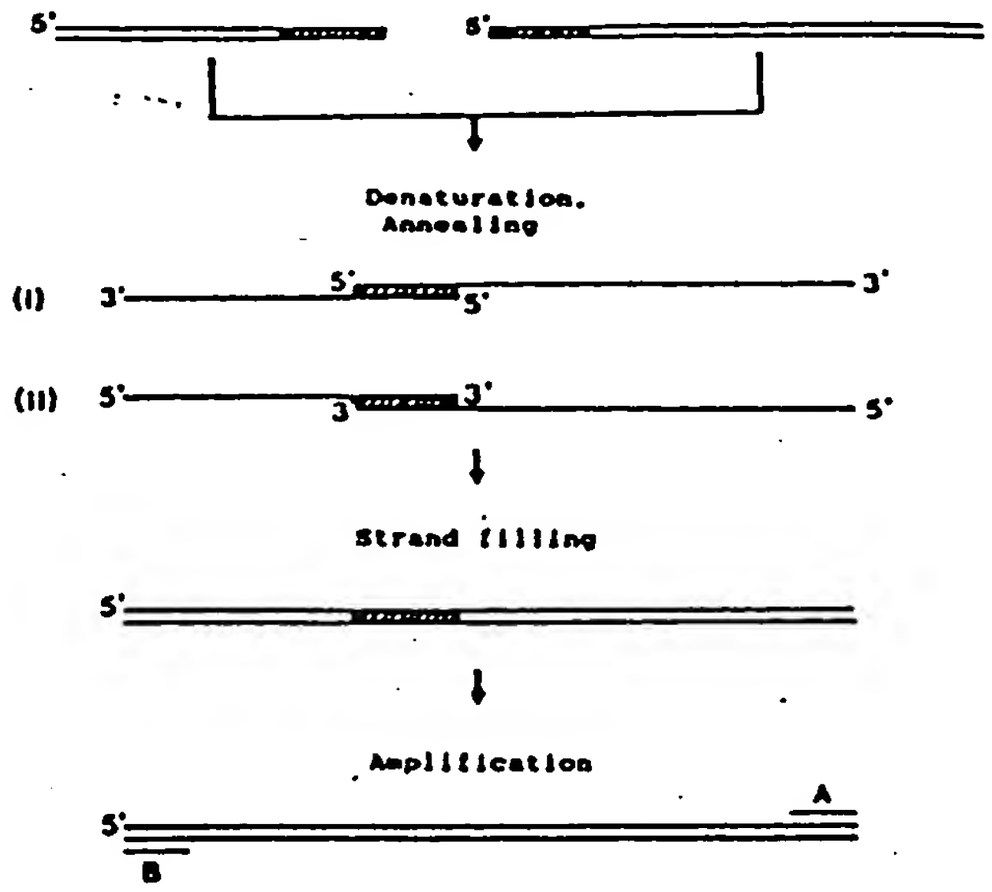

Fig. 8. Appending overlapping DNA sequences to each other. DNA molecules that overlap in a head-to-tail fashion at their respective 3' and 5' ends (e.g., partial cDNAs from the same mRNA) can be combined by denaturation, annealing and strand filling by a DNA polymerase. Amplification of such product to a preparative level is achieved using the primers *A* and *B*. Only molecules combined by annealing and completed by strand filling can be amplified. Note that of the two possible overlap annealing combinations (designated *I* and *II*) only that with overlapping 3' ends (combination *II*) can be processed further

which overlapped with the 3' ends of products made in the preceding reactions. The main use of this protocol may be not so much the in vitro synthesis of long DNA molecules (genes), but the rapid reconstruction of full-length cDNAs (e.g., for expression cloning) when only overlapping fragments are available (Fig. 8) or, alternatively, the rapid construction of chimeric genes.

In a related approach, selected subregions of regulatory or other genomic regions can be accurately isolated and, at the same time, modified by amplification, independent of appropriate restriction sites and without recourse to time-consuming processing and subcloning of DNA. Amplification not only provides sufficient quantities of target DNA for cloning, but affords, as mentioned above, the introduction of restriction sites at the ends of the fragments, allowing rapid insertion of DNA molecules into different genetic environments (e.g., expression vectors).

The generation of cDNA with mRNA as the initial template is straightforward if primer sequences can be derived from homologous genomic sequences or from a closely related heterologous sequence of a different species. If appropriate information regarding the DNA sequence is not available, known protein sequences may guide the synthesis of (degenerate) primers required for amplification. The feasibility of the protein approach has been demonstrated by Lee et al. (1988), who amplified a partial cDNA probe (112 bp) specific for the porcine enzyme urate oxidase using liver mRNA as a template. This enzyme does not exist in humans. The amplified fragment was used for the isolation of the complete porcine urate oxidase cDNA. This DNA served subsequently as a probe for the identification of regions in the human genome having homology with the porcine cDNA. Preliminary data

suggest that sequences related to this non-essential gene exist in the human genome, possibly in the form of a functionally inactive single-copy pseudogene. In addition to the significance that this observation may have for the analysis of human gene evolution, these authors have convincingly demonstrated the usefulness of the PCR for the rapid cloning of cDNA solely based on partial protein information. (A somewhat unexpected result of this experiment was the observation that mismatching of primer sequences does not necessarily preclude specific amplification; this is also of practical relevance.)

Working with degenerate primers can be facilitated by including inosine in the primer sequences at positions with a high degree of codon degeneration. Since inosine is to a certain extent neutral with respect to base pairing, use of this base improves significantly the chance that primers recognize target sequences that are only partially known (Knoth et al. 1988).

Another approach to clone cDNA can be applied if knowledge of a single short stretch of sequence within the mRNA is available. In essence, cDNAs are generated by separately amplifying two regions of the cDNA, one reaching from the known sequence to the 5' end and the other one extending to the 3' end, respectively. For both amplifications two primers are needed. One is taken from the known region within the mRNA, and the second one is complementary either to the naturally occurring poly(A) tail of the mRNA at the 3' end or to a poly(dA) tail, which is artificially attched to the 3' end of the first cDNA strand made in vitro (for details see Frohman et al. 1988). This protocol may be particularly useful if the 3' end of a mRNA has been cloned, but the 5' end is missing. In these cases the completion of a partial cDNA to a full-length cDNA can be obtained with little effort.

The relative ease with which DNAs can be obtained on a preparative scale by amplification should not conceal the caveat that exists regarding sequence fidelity (see the discussion of the technique of the PCR). Amplified and subsequently cloned DNA molecules require additional sequence verification, in particular if the DNA is intended to be used in biological studies or for protein production and protein engineering in a host system (see, e.g., Takeshita et al. 1988).

Finally, it should be noted that an intricate and very efficient scheme for RNA-dependent amplification of RNA molecules has recently been reported as an alternative to primer-dependent DNA amplification (Lizardi et al. 1988). In this reaction, Qβ replicase was used as the catalyst. The templates were recombinant transcripts made in vitro: cRNAs synthesized by T7 RNA polymerase on appropriate DNA templates. These transcripts contained at their 3' ends recognition sequences required for Qβ RNA replication (for review, see Biebricher 1983). Neither oligonucleotide priming nor cycling between different temperatures was needed. Since both RNA template and product strands are accepted for the initiation of a new round of replication, the increase in the amount of product is (as for the DNA-dependent PCR) exponential. The reaction is restricted to (engineered) RNA molecules that include Qβ-specific signals. This type of amplification may be a powerful method for the generation of RNA probes, which at the same time can be used as amplifiable "reporter" molecules (i.e., they could indicate the presence of target DNA sequences in membrane-immobilized heterogeneous mixtures). Whether this procedure is suitable as a general method for the amplification of RNA is as yet not known. The answer depends critically on the effects that non-Qβ-RNA sequences covalently linked to Qβ-replication signals have on the activity of Qβ replicase.

Conclusions and perspectives

The PCR with its enormous increase in the sensitivity of the analysis of small amounts of DNA or RNA does not replace existing molecular methods, but rather adds, by its superior resolution properties, to the potentials of established or newly developed procedures (e.g., Myers and Maniatis 1986; Church and Kieffer-Higgins 1988; Landegren et al. 1988).

The concept of the PCR is theoretically straightforward and many successful experiments have been reported, but it is not fully established with respect to all of its aspects. Limits in its applicability exist at the moment regarding target length and sequence fidelity. The latter complication may turn out to be a somewhat serious drawback of the method unless conditions can be defined in which the rate of misincorporations can be substantially reduced. In exceptional cases, secondary structure of template molecules (e.g., inverted repeats) may block synthesis to an extent which constrains amplification. Although it has been reported that extensive purification of crude nucleic acid extracts is not necessary, this may not always be the case. If perfect matching of primers is not required for an amplification to be specific, competing reactions in crude mixtures may complicate the results. It is obvious that, because some experimental standardization is still lacking, PCR is not yet a routine procedure for diagnostic purposes. In addition, the reaction is rather expensive for the daily use.

Nevertheless, it is already quite clear from numerous published results that most conditions (incubation times, concentrations of reaction components, temperatures for annealing and synthesis, lengths and base compositions of primers, etc.) can be tailored to suit very different analytical and preparative purposes. Thus, the PCR will become an advanced and fairly general technique for the study of genes and gene activities.

The typical diagnostic application in genetics will be the DNA or RNA sequence, which is altered by mutation, polymorphic variation, deletion, translocation, recombination or related processes. In addition, extensive gene dosage variations caused, e.g., by repeated duplication of genes in the genome (this process is also called amplification), may easily be recognized. Loss or gain of whole chromosomes (mono- or trisomies) and major rearrangements are probably more reliably recognized by recently developed cytogenetic techniques (designated in situ suppression hybridization), which allow the visualization of chromosomes and subchromosomal regions with a very high resolution even in interphase nuclei (Cremer et al. 1988; Lichter et al. 1988).

The importance of PCR for the progress of genetic research will in the long run be significant in more than one respect. A few points regarding possible developments may be emphasized.

1. With regard to molecular biology, amplification will contribute to the rate of DNA sequence acquisition. Furthermore, gene activity studies will benefit from the analysis of very few cells and ultimately single cells. Finally, gene targeting may gain ground with the availability of a sensitive and relatively fast method for the monitoring of recombinational success in cases not amenable to selection. It is even conceivable that, in

conjuction with elaborate microinjection techniques (Ansorge and Pepperkok 1988), new gene replacement strategies can be designed.

2. The analysis of single sperm offers the chance of constructing an improved recombination map of the human genome. PCR-derived data will eventually complement information that is currently being collected by other powerful molecular techniques, such as pulse-field gradient gel electrophoresis (PFGE) (Schwartz and Cantor 1984) and chromosomal jumping (Poustka et al. 1987).

3. The intensity of diagnostic activities in genetic research will generally increase. It is quite obvious that PCR will guide the mutation analysis of identified disease genes by allowing direct sequencing of DNA. Since amplification of genomic DNA does not depend on the presence of restriction sites, the term "RFLP" can be considered as describing a special case of the general phenomenon "DNA sequence polymorphism" (DSP, D. W. Yandell, personal communication). Screening, at least for certain genetic traits, may become feasible in the general population in addition to families at risk. Advanced knowledge will become available about the causes and pathogenic mechanisms of multifactorial disorders or, more specifically, about the contributions of genetic polymorphisms to common diseases. The concept of risk prediction may come one step closer to its realization. (Considering the potential of PCR, we may approach the point where we have to decide how much prediction we want.)

4. As a consequence of this development, a tight link will develop between human genetic research (including clinical genetics) and basic molecular biology. Progress in human genetics is largely based on the observation of random events in non-selectively breeding populations rather than on deliberate experimentation. The possibility of extending the range of observations to cells, molecules and genes of individual persons and of comparing them with each other will significantly advance our concepts of human biology and genetics [see the recent discussion by White and Caskey (1988) with regard to the human as an experimental system in molecular genetics].

This list is certainly incomplete, and one might argue as to where the main emphasis should be placed. Additional relevant applications in both medical science and molecular biology are likely; these will eventually confirm the PCR as a major technical breakthrough.

Acknowledgements. The critical reading of the manuscript and many suggestions by Drs. G. Czihak (Salzburg) and W. Krone (Ulm) are gratefully acknowledged.

References

Abbott CM, McMahon CJ, Whitehouse DB, Povey S (1988) Prenatal diagnosis of alpha-1-antitrypsin deficiency using polymerase chain reaction. Lancet 1:763–764

Amselem S, Nunes V, Vidaud M, Estivill X, Wong C, Dauriol L, Vidaud D, Galibert F, Baiget M, Goossens M (1988) Determination of the spectrum of β-thalassemia genes in Spain by use of dot-blot analysis of amplified β-globin DNA. Am J Hum Genet 43:95–100

Ansorge W, Pepperkok R (1988) Performance of an automated system for capillary microinjection into living cells. J Biochem Biophys Methods 16:283–292

Aquadro CF, Greenberg BD (1983) Human mitochondrial DNA variation and evolution: analysis of nucleotide sequences from seven individuals. Genetics 103:287–312

Beck BN, Ho SN (1988) Increased specifity of PCR-amplified products by size-fractionation of restriction enzyme-digested template genomic DNA. Nucleic Acids Res 16:9051

Berg K (1986) Normal genetic lipoprotein variations and atherosclerosis. In: Sirtori CR, Nichols AV, Franceschini G (eds) Human apolipoprotein mutants. Plenum Press, New York, pp 31–49

Bhagavati S, Ehrlich G, Kula RW, Kwok S, Sninsky J, Udani V, Poiesz BJ (1988) Detection of human T-cell lymphoma/leukemia virus type I DNA and antigen in spinal fluid and blood of patients with chronic progressive myelopathy. N Engl J Med 318:1141–1147

Biebricher CK (1983) Darwinian selection of self-replicating RNA molecules. Evol Biol 16:1–52

Bos JL, Fearon ER, Hamilton SR, Verlaan-de Vries M, Boom JH van, Eb AJ van der, Vogelstein B (1987) Prevalence of *ras* gene mutations in human colorectal cancers. Nature 327:293–297

Botstein D, White RL, Skolnick M, Davis RW (1980) Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am J Hum Genet 32:314–331

Bruun Petersen K, Koelvraa S, Bolund L, Bruun Petersen G, Koch J, Gregersen N (1988) Detection of alpha1-antitrypsin genotypes by analysis of amplified DNA sequences. Nucleic Acids Res 16:352

Bugawan TL, Saiki RK, Levenson CH, Watson RM, Erlich HA (1988) The use of non-radioactive oligonucleotide probes to analyze enzymatically amplified DNA for prenatal diagnosis and forensic HLA typing. BioTechnology 6:943–947

Byrne BC, Li JJ, Sninsky J, Poiesz B (1988) Detection of HIV-1 RNA sequences by in vitro DNA amplification. Nucleic Acids Res 16:4165

Cai S-P, Zhang J-Z, Huang D-H, Wang Z-X, Kan YW (1988) A simple approach to prenatal diagnosis of β-thalassemia in a geographic area where multiple mutations occur. Blood 71:1357–1360

Cariello NF, Scott JK, Kat AG, Thilly WG, Keohavong P (1988) Resolution of a missense mutant in human genomic DNA by denaturing gradient gel electrophoresis and direct sequencing using in vitro DNA amplification: HPRT Munich. Am J Hum Genet 42:726–734

Chamberlain JS, Gibbs RA, Ranier JE, Nguyen PN, Caskey CT (1988) Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. Nucleic Acids Res 16:11141–11156

Chan V, Chan TK, Kan YW, Todd D (1988) A novel β-thalassemia frameshift mutation (codon 14/15), detectable by direct visualization of abnormal restriction fragment in amplified genomic DNA. Blood 72:1420–1423

Chehab FF, Doherty M, Cai S, Kan YW, Cooper S, Rubin EM (1987) Detection of sickle cell anemia and thalassemias. Nature 329:293–294

Chelly J, Kaplan J-C, Maire P, Gautron S, Kahn A (1988) Transcription of the dystrophin gene in human muscle and non-muscle tissues. Nature 333:858–860

Chien A, Edgar DB, Trela JM (1976) Deoxyribonucleic acid polymerase from the extreme thermophile *Thermus aquaticus*. J Bacteriol 127:1550–1557

Church GM, Kieffer-Higgins S (1988) Multiplex DNA sequencing. Science 240:185–188

Connor BJ, Reyes AA, Morin C, Itakura K, Teplitz RL, Wallace RB (1983) Detection of sickle cell β^s-globin allele by hybridization with synthetic oligonucleotides. Proc Natl Acad Sci USA 80:278–282

Cremer T, Lichter P, Borden J, Ward DC, Manuelidis L (1988) Detection of chromosome aberrations in metaphase and interphase tumor cells by in situ hybridization using chromosome-specific library probes. Hum Genet 80:235–246

Crescenzi M, Seto M, Herzig GP, Weiss PD, Griffith RC, Korsmeyer SJ (1988) Thermostable DNA polymerase chain amplification of T(14–18) chromosome breakpoints and detection of minimal residual disease. Proc Natl Acad Sci USA 85:4869–4873

C (1988) A sensitive non-radioactive PCR-RFLP analysis for detecting point mutations at 12th codon of oncogene c-Ha-ras in DNAs of gastric cancer. Nucleic Acids Res 16:6231

Denton PH, Fowlkes DM, Lord ST, Reisner HM (1988) Hemophilia-B Durham – a mutation in the first EGF-like domain of factor-IX that is characterized by polymerase chain reaction. Blood 71: 1407–1411

Diaz-Chico JC, Yang K, Yang K, Efremov DG, Stoming TA, Huisman THJ (1988) The detection of β-globin gene mutations in β-thalassemia using oligonucleotide probes and amplified DNA. Biochim Biophys Acta 949:43–48

DiLella AG, Huang W-M, Woo SLC (1988) Screening for phenylketonuria mutations by DNA amplification with polymerase chain reaction. Lancet 1:497–499

Drake JW, Baltz RH (1976) The biochemistry of mutagenesis. Annu Rev Biochem 45:11–37

Duggan DB, Ehrlich GD, Davey FP, Kwok S, Sninsky J, Goldberg J, Baltrucki L, Poiesz BJ (1988) HTLV-I-induced lymphoma mimicking Hodgkin's disease. Diagnosis by polymerase chain reaction amplification of specific HTLV-I sequences in tumor DNA. Blood 71:1027–1032

Dunning AM, Talmud P, Humphries SE (1988) Errors in the polymerase chain reaction. Nucleic Acids Res 16:10393

Embury SH, Scharf SJ, Saiki RK, Gholson MA, Golbus M, Arnheim N, Erlich HA (1987) Rapid prenatal diagnosis of sickle cell anemia by a new method of DNA analysis. N Engl J Med 316: 656–661

Engelke DR, Hoener PA, Collins FS (1988) Direct sequencing of enzymatically amplified human genomic DNA. Proc Natl Acad Sci USA 85:544–548

Farr CJ, Saiki RK, Erlich HA, McCormick F, Marshall CJ (1988) Analysis of *ras* gene mutations in acute myeloid leukemia by polymerase chain reaction and oligonucleotide probes. Proc Natl Acad Sci USA 85:1629–1633

Farzadegan H, Polis MA, Wolinsky SM, Rinaldo CR, Sninsky JJ, Kwok S, Griffith RL, Kaslow RA, Phair JP, Polk F, Saah AJ (1988) Loss of human immunodeficiency virus type I (HIV-1) antibodies with evidence of viral infection in asymptomatic homosexual men. Ann Intern Med 108:785–790

Feldman GL, Williamson R, Beaudet AL, O'Brien WE (1988) Prenatal diagnosis of cystic fibrosis by DNA amplification for detection of KM-19 polymorphism. Lancet II:102

Foulkes NS, Pandolfi de Rinaldis PP, Macdonnel J, Cross NCP, Luzzatto L (1988) Polymerase chain reaction automated at low cost. Nucleic Acids Res 16:5687–5688

Frohman MA, Dush MK, Martin GR (1988) Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc Natl Acad Sci USA 85:8998–9002

Gennari K, Wermuth B, Muellener D, Ehrig T, Wartburg J-P von (1988) Genotyping of human class I alcohol dehydrogenase. Analysis of enzymatically amplified DNA with allele-specific oligonucleotides. FEBS Lett 228:305–309

Gish G, Eckstein F (1988) DNA and RNA sequence determination based on phosphorothioate chemistry. Science 240:1520–1522

Gu XF, Elion J, Ouagued M, Clauser E, Assan R, Krishnamoorthy R (1988) A simple strategy to amplify specifically the HLA-DQβ gene region with genomic DNA as template. FEBS Lett 236:23–26

Gusella JF (1986) DNA polymorphism and human disease. Annu Rev Biochem 55:831–854

Gyllensten UB, Erlich HA (1988) Generation of single-stranded DNA by the polymerase chain reaction and its application to direct sequencing of the HLA-DQA locus. Proc Natl Acad Sci USA 85:7652–7656

Haqqi TM, Sarkar G, David CS, Sommer SS (1988) Specific amplification with PCR of a refractory segment of genomic DNA. Nucleic Acids Res 16:11844

Harbarth P, Vosberg H-P (1988) Enzymatic amplification of myosin heavy-chain mRNA sequences in vitro. DNA 7:297–306

Hayes W (1974) The genetics of bacteria and their viruses, 2nd edn. Blackwell, Oxford, pp 193–199

Higuchi R, Beroldingen CH von, Sensabaugh GF, Erlich HA (1988a) DNA typing from single hairs. Nature 332:543–546

Higuchi R, Krummel B, Saiki RK (1988b) A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucleic Acids Res 15: 7351–7367

Howley PM (1987) The role of papilloma virus in human cancer. In: DeVita Jr VT, Hellman SS, Rosenberg SA (eds) Important advances in oncology. Lippincott, Philadelphia, pp 55–73

Impraim CC, Saiki RK, Erlich HA, Teplitz RL (1987) Analysis of DNA extracted from formalin-fixed, paraffin-embedded tissues by enzymatic amplification and hybridization with sequence-specific oligonucleotides. Biochem Biophys Res Commun 142:710–716

Innis MA, Myambo KB, Gelfand DH, Brow MAD (1988) DNA sequencing with *Thermus aquaticus* DNA polymerase and direct sequencing of polymerase chain reaction amplified DNA. Proc Natl Acad Sci USA 85:9436–9440

Janssen JWG, Lyons J, Steenvorden ACM, Seliger H, Bartram CR (1987a) Concurrent mutations in two different ras genes in acute myelocytic leukemias. Nucleic Acid Res 15:5669–5680

Janssen JWG, Steenvorden ACM, Lyons J, Anger B, Böhlke JU, Bos JL, Seliger H, Bartram CR (1987b) *Ras* gene mutations in acute and chronic myelotic leukemias, chronic myeloproliferative disorders, and myelodysplastic syndromes. Proc Natl Acad Sci USA 84:9228–9232

Jeffreys AJ, Wilson V, Thein SL (1985) Hypervariable "minisatellite" regions in human DNA. Nature 314:67–73

Jeffreys AJ, Wilson V, Neumann R, Keyte J (1988) Amplification of human minisatellites by the polymerase chain reaction: towards DNA fingerprinting of single cells. Nucleic Acids Res 16:10953–10971

Kaufman JF, Auffray C, Korman AJ, Shackelford DA, Strominger J (1984) The class II molecules of the human and murine major histocompatibility complex. Cell 36:1–13

Kawasaki ES, Clark SS, Coyne MY, Smith SD, Champlin R, Witte ON, McCormick FP (1988) Diagnosis of chronic myeloid and acute lymphocytic leukemias by detection of leukemia-specific mRNA sequences amplified in vitro. Proc Natl Acad Sci USA 85: 5698–5702

Keohavong P, Kat AG, Cariello NF, Thilly WG (1988a) DNA amplification in vitro using T4 DNA polymerase. DNA 7:63–70

Keohavong P, Wang CC, Cha RS, Thilly WG (1988b) Enzymatic amplification and characterization of large DNA fragments from genomic DNA. Gene 71:211–216

Kim H-S, Smithies O (1988) Recombinant fragment assay for gene targetting based on the polymerase chain reaction. Nucleic Acids Res 16:8887–8903

Knoth K, Roberds S, Poteet C, Tamkun M (1988) Highly degenerate, inosine-containing primers specifically amplify rare cDNA using the polymerase chain reaction. Nucleic Acids Res 16:10932

Koenig M, Hoffman EP, Bertelson CJ, Monaco AP, Feener C, Kunkel LM (1987) Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. Cell 50:509–517

Kogan SC, Doherty M, Gitschier J (1987) An improved method for prenatal diagnosis of genetic diseases by analysis of amplified DNA sequences. N Engl J Med 317:985–990

Kozma SC, Bogaard ME, Buser K, Saurer SM, Bos JL, Groner B, Hynes NE (1987) The human c-Kirsten *ras* gene is activated by a novel mutation in codon 13 in the breast carcinoma cell line MDA-MB231. Nucleic Acids Res 15:5963–5971

Kuchta RD, Benkovic P, Benkovic SJ (1988) Kinetic mechanism whereby DNA polymerase I (Klenow) replicates DNA with high fidelity. Biochemistry 27:6716–6725

Kulozik AE, Lyons J, Kohne E, Seliger H, Bartram CR, Kleihauer E (1988) Rapid and non-radioactive prenatal diagnosis of β-thalassemia and sickle cell disease: application of the polymerase chain reaction (PCR). Br J Haematol 70:455–458

Kwok S, Mack DH, Mullis KB, Poiesz B, Ehrlich G, Blair D, Friedman-Kien A, Sninsky JJ (1987) Identification of human immunodeficiency virus sequences by using in vitro enzymatic amplification and oligomer cleavage detection. J Virol 61:1690–1694

Lai-Goldman M, Lai E, Grody WW (1988) Detection of human immunodeficiency virus (HIV) infection in formalin fixed, paraffin

embedded tissues by DNA amplification. Nucleic Acids Res 16: 8191

Landegren U, Kaiser R, Caskey CT, Hood L (1988) DNA diagnostics – molecular techniques and automation. Science 242:229–237

Larzul D, Guigue F, Sninsky JJ, Mack DH, Brechot C, Guesdon JL (1988) Detection of hepatitis B virus sequences in serum by using in vitro enzymatic amplification. J Virol Methods 20:227–237

Layton DM, Mufti GJ, Lyons J, Janssen JWG, Bartram CR (1988) Loss of *ras* oncogene mutation in a myelodysplastic syndrome after low-dose cytarabine therapy. Lancet I:1468–1469

Lee CC, Wu X, Gibbs RA, Cook RG, Muzny DM, Caskey CT (1988) Generation of cDNA probes directed by amino acid sequence: cloning of urate oxidase. Science 239:1288–1291

Lee M-S, Chang K-S, Cabanillas F, Freireich EJ, Trujillo JM, Stass SA (1987) Detection of minimal residual cells carrying the t(14;18) by DNA sequence amplification. Science 237:175–178

Levinson B, Janco R, Philipps III J, Gitschier J (1987) A novel missense mutation in the factor VIII gene identified by analysis of amplified hemophilia DNA sequences. Nucleic Acids Res 15: 9797–9805

Li H, Gyllensten UB, Cui X, Saiki RK, Erlich HA, Arnheim N (1988) Amplification and analysis of DNA sequences in single human sperm and diploid cells. Nature 335:414–417

Liang W, Johnson JP (1988) Rapid plasmid insert amplification with polymerase chain reaction. Nucleic Acids Res 16:3579

Lichter P, Cremer T, Manuelidis L, Ward DC (1988) Delineation of individual chromosomes in metaphase and interphase cells by in situ suppression hybridization using recombinant DNA libraries. Hum Genet 80:224–234

Lizardi PM, Guerra CE, Lomeli H, Tussie-Luna I, Kramer FR (1988) Exponential amplification of recombinant-RNA hybridization probes. BioTechnology 6:1197–1202

Lo Y-MD, Mehal WZ, Fleming KA (1988a) Rapid production of vector-free biotinylated probes using the polymerase chain reaction. Nucleic Acids Res 16:8719

Lo Y-MD, Mehal WZ, Fleming KA (1988b) False-positive results and the polymerase chain reaction. Lancet II:679

Lyons J, Janssen JWG, Bartram C, Layton M, Mufti GJ (1988) Mutation of Ki-*ras* and N-*ras* oncogenes in myelodysplastic syndromes. Blood 71:1707–1712

Marshall CJ (1986) The *ras* gene family. In: Kuhn P, Graf T (eds) Oncogenes and growth control. Springer, Berlin Heidelberg New York, pp 192–199

Maxam A, Gilbert W (1980) Sequencing end-labelled DNA with base specific chemical cleavage. Methods Enzymol 65:499–560

McConlogue L, Brow MAD, Innis MA (1988) Structure-independent DNA amplification by PCR using 7-deaza-2'-deoxyguanosine. Nucleic Acids Res 16:9869

McMahon G, Davis E, Wogan GN (1987) Characterization of c-Ki-*ras* oncogene alleles by direct sequencing of enzymatically amplified DNA from carcinogen-induced tumors. Proc Natl Acad Sci USA 84:4974–4978

Mitchell GA, Brody LC, Looney J, Steel G, Suchanek M, Dowling C, Kaloustian VD, Kaiser-Kupfer M, Valle D (1988) An initiator codon mutation in ornithine-δ-aminotransferase causing gyrate atrophy of the choroid and retina. J Clin Invest 81:630–633

Mullis KB, Faloona FA (1987) Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods Enzymol 155:335–350

Mullis K, Faloona F, Scharf S, Saiki R, Horn G, Erlich H (1986) Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harbor Symp Quant Biol 51:263–273

Murakawa GJ, Zaia JA, Spallone PA, Stephens DA, Kaplan BE, Wallace RB, Rossi JJ (1988) Direct detection of HIV-1 RNA from AIDS and ARC patient samples. DNA 7:287–295

Myers RM, Maniatis T (1986) Recent advances in the development of methods for detecting single-base substitutions associated with human genetic diseases. Cold Spring Harbor Symp Quant Biol 51:275–284

Myers RM, Larin Z, Maniatis T (1985) Detection of single base substitutions by ribonuclease cleavage at mismatches in RNA:DNA duplexes. Science 230:1242–1246

Nakamaye KL, Gish G, Eckstein F, Vosberg H-P (1988) Direct sequencing of polymerase chain reaction amplified DNA fragments through the incorporation of deoxynucleoside α-thiotriphosphates. Nucleic Acids Res 16:9947–9959

Newton CR, Kalsheker N, Graham A, Powell S, Gammack A, Riley J, Markham AF (1988) Diagnosis of α_1-antitrypsin deficiency by enzymatic amplification of human genomic DNA and direct sequencing of polymerase chain reaction products. Nucleic Acids Res 16:8233–8243

Noonan KE, Roninson IB (1988) mRNA phenotyping by enzymatic amplification of randomly primed cDNA. Nucleic Acids Res 16: 10366

Oste C (1988) Polymerase chain reaction. BioTechniques 6:162–167

Ou C-Y, Kwok S, Mitchell SW, Mack DH, Sninsky JJ, Krebs JW, Feorino P, Warfield D, Schochetman G (1988) DNA amplification for direct detection of HIV-1 in DNA of peripheral blood mononuclear cells. Science 239:295–297

Pääbo S, Wilson AC (1988) Polymerase chain reaction reveals cloning artefacts. Nature 334:387–388

Poustka A, Pohl TM, Barlow DP, Frischauf A-M, Lehrach H (1987) Construction and use of human chromosome jumping libraries from *Not*I-digested DNA. Nature 325:353–355

Powell LM, Wallis SC, Pease RJ, Edwards YH, Knott TJ, Scott J (1987) A novel form of tissue-specific RNA processing produces apolipoprotein-B48 in intestine. Cell 50:831–840

Rappolee DA, Mark D, Banda MJ, Werb Z (1988) Wound macrophages express TGF-α and other growth factors in vivo: analysis by mRNA phenotyping. Science 241:708–712

Rochlitz CF, Scott GK, Dodson JM, Benz CC (1988) Use of the polymerase chain reaction technique to create base-specific *ras* oncogene mutations. DNA 7:515–519

Rollo F, Amici A, Salvi R (1988) A simple and low cost DNA amplifier. Nucleic Acids Res 16:3105

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N (1985) Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230:1350–1354

Saiki RK, Bugawan TL, Horn GT, Mullis KB, Erlich HA (1986) Analysis of enzymatically amplified β-globin and HLA-DQα DNA with allele specific oligonucleotide probes. Nature 324:163–166

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988a) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487–494

Saiki RK, Chang CA, Levenson CH, Warren TC, Boehm CD, Kazazian HH, Erlich HA (1988b) Diagnosis of sickle-cell anemia and β-thalassemia with enzymatically amplified DNA and nonradioactive allele-specific oligonucleotide probes. N Engl J Med 319:537–541

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain termination inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Sarkar G, Sommer SS (1988) RNA amplifications with transcript sequencing (RAWTS). Nucleic Acids Res 16:5197

Scharf SJ, Horn GT, Erlich HA (1986) Direct cloning and sequence analysis of enzymatically amplified genomic sequences. Science 233:1076–1078

Scharf SS, Friedmann A, Brautbar C, Szafer F, Steinman L, Horn G, Gyllensten U, Erlich HA (1988) HLA class II allelic variation and susceptibility to pemphigus vulgaris. Proc Natl Acad Sci USA 85: 3504–3508

Schwartz DC, Cantor CR (1984) Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. Cell 37: 67–75

Shibata DK, Arnheim N, Martin WJ (1988) Detection of human papilloma virus in paraffin-embedded tissue using the polymerase chain reaction. J Exp Med 167:225–230

Simpson D, Crosby RM, Skopek TR (1988) A method for specific cloning and sequencing of human HPRT cDNA for mutation analysis. Biochem Biophys Res Commun 151:487–492

Smit VTHBM, Boot AJM, Smits AMM, Fleuren GJ, Cornelisse CJ, Bos JL (1988) KRAS codon 12 mutations occur very frequently in pancreatic adenocarcinomas. Nucleic Acids Res 16:7773–7782

mithies O, Gregg RG, Boggs SS, Koralewski MA, Kucherlapati RS (1985) Insertion of DNA sequences into the human chromosomal β-globin locus by homologous recombination. Nature 317:230–234

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517

Stoflet ES, Koeberl DD, Sarkar G, Sommer SS (1988) Genomic amplification with transcript sequencing. Science 239:491–494

Syvänen AC, Bengtström M, Tenhunen J, Söderlund H (1988) Quantification of polymerase chain reaction products by affinity-based hybrid collection. Nucleic Acids Res 16:11327–11338

Takeshita S, Tezuka K, Takahashi M, Honkawa H, Matsuo A, Matsuishi T, Hashimoto-Goto T (1988) Tandem gene amplification in vitro for rapid and efficient expression in animal cells. Gene 71:9–18

Teynor-Maclachlan A (1988) Versuche zur Optimierung der PCR-Methode. Thesis, University of Heidelberg

Tindall KR, Kunkel TA (1988) Fidelity of DNA synthesis by the *Thermus aquaticus* DNA polymerase. Biochemistry 27:6008–6013

Todd JA, Bell JI, McDevitt HO (1987) HLA-DQβ gene contributes to susceptibility and resistance to insulin-dependent diabetes mellitus. Nature 329:599–604

Triglia T, Peterson MG, Kemp DJ (1988) A procedure for in vitro amplification of DNA segments that lie outside the boundaries of known sequences. Nucleic Acids Res 16:8186

Veer LJ van't, Hermens R, Berg-Bakker LAM van den, Cheng NC, Fleuren G-J, Bos JL, Cleton FJ, Schrier PI (1988) *ras* Oncogene activation in human ovarian carcinoma. Oncogene 2:157–165

Veres G, Gibbs RA, Scherer SE, Caskey CT (1987) The molecular basis of the sparse fur mouse mutation. Science 237:415–417

Verlaan-de Vries M, Bogaard ME, Elst H van den, Boom J van, Eb A van der, Bos JL (1986) A dot-blot screening procedure for mutated *ras* oncogenes using synthetic oligonucleotides. Gene 50:313–320

Vigilant L, Stoneking M, Wilson AC (1988) Conformational mutation in human mtDNA detected by direct sequencing of enzymatically amplified DNA. Nucleic Acids Res 16:5945–5955

Weisgraber KH, Newhouse YM, Mahley RW (1988) Apolipoprotein E genotyping using the polymerase chain reaction and allele-specific oligonucleotide probes. Biochem Biophys Res Commun 157:1212–1217

White R, Caskey CT (1988) The human as an experimental system in molecular genetics. Science 240:1483–1488

Williams C, Williamson R, Coutelle C, Loeffler F, Smith J, Ivinson A (1988) Same-day, first-trimester antenatal diagnosis for cystic fibrosis by gene amplification. Lancet II:102–103

Wong C, Dowling CE, Saiki RK, Higuchi RG, Erlich HA, Kazazian Jr HH (1987) Characterization of β-thalassemia mutations using direct genomic sequencing of amplified single copy DNA. Nature 330:384–386

Wrischnik LA, Higuchi RG, Stoneking M, Erlich HA, Arnheim N, Wilson AC (1987) Length mutations in human mitochondrial DNA: direct sequencing of enzymatically amplified DNA. Nucleic Acids Res 15:529–542

Received December 22, 1988